ON THE STRUCTURE AND NATURE OF THE ENDOSPORE IN STRAIN C_3 OF BACILLUS CEREUS

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The early literature on the structural organization of the endospore and on the cytological processes involved in its formation and germination was reviewed by Knaysi (1948). Since then a number of articles variously dealing with these subjects have appeared (briefly discussed by Robinow, 1953*a*, *b*). A careful study of this recent literature reveals that, in spite of the considerable progress achieved, much remains to be learned. For instance, not much is known about the nucleus which initiates endospore formation (Knaysi, 1952), nor is there yet general agreement on the structure of the endospore or on some of the intimate processes which accompany its germination (*cf* Bisset and Hale, 1951; Bisset, 1952; DeLamater and Hunter, 1952; Hunter and DeLamater, 1952; Robinow, 1953*a*; Fitz-James, 1953). The difficulty in the way of a clear understanding of spore structure has been not only the formation around the spore of a coat of very low permeability, but also the fact that the forespore stains deeply and uniformly by the methods commonly used to demonstrate the nucleus. Both difficulties have been overcome (Robinow, 1953*b*) by cutting the spore into thin sections. It is significant that study of these thin sections led Robinow to consider the endospore as "a specialized nucleus, distended by and encased in products of its own activity", a view which he considers as approximating the description of spore formation by the author (Knaysi, 1952).

The present investigation is a corollary to one in which we investigated the structure of the bacterial nucleus (Knaysi, 1955). It was possible to develop a method (method *C*) highly suitable for studying the structural organization of the forespore (figures 1 to 3 and 13 to 15) as well as that of the germinating spore at a very early stage in the process (figures 8 to 11).

MATERIALS AND METHODS

Practically all observations were made on strain C_3 of *Bacillus cereus* grown on collodion membranes supported by various agar media (Hillier, Knaysi, and Baker, 1948). In most cases the supporting medium had the following composition: 0.2 g of glucose + 0.2 g of Na acetate + 2 g of agar + 100 ml of distilled water (medium GA), but for the study of spore germination we often used media containing 0.1 g of glucose + 0.1 g of yeast extract powder in addition to agar and water as above, or consisting of half-strength, beef infusion + 0.5 g of glucose + 0.5 g of tryptone + 2 g of agar per 100 ml of infusion (medium MITG/2). Most commonly, the incubation temperature was 30 C.

At the proper age a colony is cut off the agar plate and floated with the supporting membrane on distilled water whence it is picked up with a cover glass, placed in an inclined position, and allowed to dry in the air. Drying may be hastened by absorbing the water that drains to the edge of the collodion film with a strip of blotting paper. The air-dry colony is then fixed for 2 to 5 minutes with alcohol or for 1 minute with formalin vapors. Formalin fixation was preferable to alcohol fixation for the study of germination and of the vegetative stage in general. Before or after fixation one may draw two perpendicular diameters of the colony with a soft, very sharp pencil. These lines may serve as axes of coordinates by means of which one can locate any particular cell or group of cells in the colony. Very often suitable cells or groups of cells may be found in the immediate vicinity of the intersection point of the diameters (origin of the axes).

The fixed colony is then mounted in a film of a slightly acidified thionin solution prepared by adding 0.5 ml of 0.1 N HCl to 9 ml of 0.1 per cent aqueous thionin solution: The cover glass carrying the culture is inverted over a droplet of the dye solution on a clean, glass slide; the excess solution is absorbed with strips of blotting paper applied to the edges of the cover glass, and the cover glass is sealed to the slide with vaspar (mixture of equal volumes of vaseline and

paraffine). The cytoplasm stains moderately, but the compound nuclei and forespores, more deeply with this dye solution. The primary nuclei usually are not clearly visible. One selects a suitable cell or a suitable group or groups of cells that one photographs. The forespores show the typical, homogeneous appearance (figures 1 and 13). Following this, the preparation is disconnected, the excess thionin solution still adhering to the cover glass is carefully absorbed with a strip of blotting paper, and the colony remounted on a fresh slide in a film of methylene blue solution of pH 3.1 to 3.2, prepared by mixing 1 ml of a 1 per cent aqueous, methylene blue solution with 1 ml of 0.1 N HCl + 2 ml of 0.1 M sodium acid phthalate + 6 ml of distilled water. The excess dye is absorbed as above, and the preparation is then sealed with vaspar and examined with the microscope. In particular, the cells or cell groups that were photographed when the colony was mounted in thionin are located, carefully studied, and rephotographed. The preparation may again be disconnected, the colony variously treated and stained, and the selected cells or cell groups studied and photographed. In particular, one may subject the colony to the Feulgen or the HCl-Giemsa procedures, to Gram's reaction, to enzymatic treatment, etc. The effects of the various treatments on the same cells may then be compared (figures 1 to 3 and 13 to 15).

The photomicrographs were taken on microfile film using a Contax camera with special attachment to the microscope. Wratten filters, single or in combination, were used to produce sharper images and maximum contrast. The exposed films were developed with microdol.

RESULTS

The method used is superior to any heretofore known method for the demonstration of primary nuclei and of the structure of the forespore. It has only a limited advantage over acidified methylene blue alone for studying the structure of the compound nucleus. In the latter case, fixation with formalin vapors for 3 or more minutes seems to be of some help in partially unmasking the chromatin of the compound nucleus (Knaysi, 1955). The present work shows that the forespore is formed by a compound nucleus in which the nucleoplasm appears to have undergone a change in staining properties. This change consists in a loss of metachromasy and a rise in isoelectric point to the neighborhood of that of common proteins.

The structure of the forespore presents some variation but may be resolved into three general types. In the first type it appears as if the complex nucleus had broken up into its component, primary nuclei, usually each containing two terminal beads or chromosomes. Most commonly one finds two nuclei, parallel (figures 4, *a* to *d*; 6, *a* to *c*) or intersecting (figures 6, *d*, and 7, *a*, *b*); three or, occasionally, four or six primary nuclei are also found (figures 7, *c*; 12, *d* to *f*; 14, *a*). In the second type there is no suggestion of structural reorganization. The forespore appears surrounded by a beaded string of chromatin (figure 5, *b*) which, in reality, is a complex primary nucleus resulting either from growth and division of the chromosomes without division of the nucleus or from fusion, end to end, of simple primary nuclei. The third type is intermediate. The forespore appears to be laterally bound by two primary nuclei, one at each side. At least one of these nuclei contains three chromosomes. Sometimes both nuclei contain three chromosomes each (figure 12, *a* to *c*).

The method used in this investigation permits observation of the internal structure of the endospore at a very early stage of germination (figures 8 to 11). It is indeed possible to observe the germinating spore before it undergoes any noticeable change in internal structure. At such an early stage the germinating spore is found to have the same internal structure as the forespore (figures 8, *a* and *b*; 10, *a* to *f*), indicating that the internal structure of the endospore is identical to that of the forespore. At later stages, the primary nuclei begin to elongate and divide; the complex primary nuclei break up into simple ones that also elongate and divide. In all cases this activity is accompanied by a centripetal shift in the position of these nuclei; they move toward the center of what is now the cytoplasm of the germ cell and what was the nucleoplasm of the forespore, and before that the nucleoplasm of the compound nucleus which formed the forespore. Under good conditions of growth, the primary nuclei grow and divide faster than does the cell so that these nuclei appear in groups (figures 8 *c* to *e*; 9; 11, *a*). These groups alone or in relation to other groups present in the same cell have been interpreted by DeLamater and Mudd (1951) as nuclei of the type found in higher plants and animals at

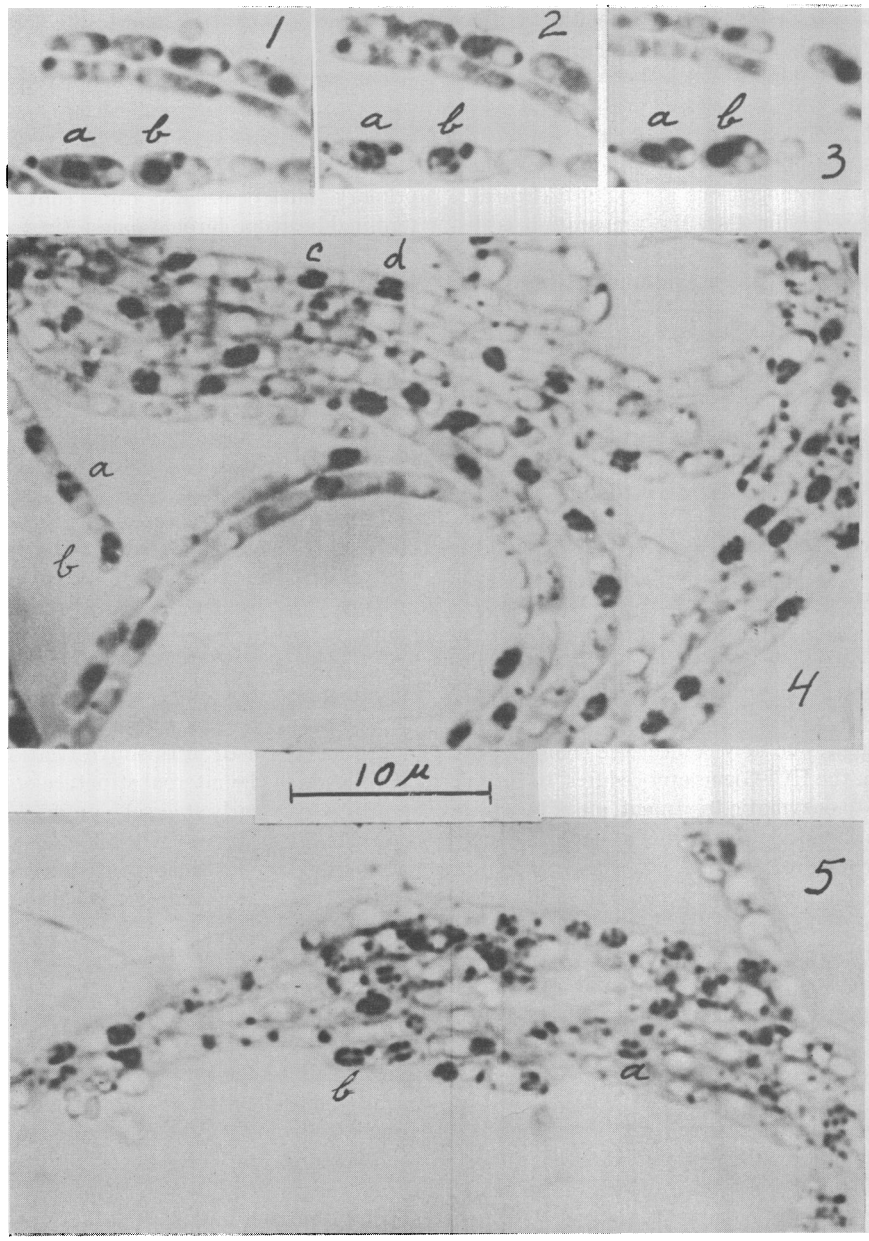

Figure 1. (54 F-18). A microculture on collodion film supported by GA agar. Age 24 hr at 30 C. Fixed with ethanol and mounted in acid thionin. Bodies *a* and *b* are forespores.

Figure 2. (54 F-21). The same cells as in figure 1 remounted in methylene blue solution of pH 3.1 to 3.2. Note that forespores *a* and *b* which appeared homogeneous in figure 1 now show structural differentiation.

Figure 3. (54 F-24). The same cells as in figures 1 and 2 after treatment with N HCl at 60 C for 10 min and restaining with Giemsa's solution diluted 5 times with distilled water. Note that forespores *a* and *b* stain homogeneously by this method. Note also some change in the morphology of these forespores as compared to figure 1.

Figure 4. (54 E-23). A microculture on collodion film supported by GA agar. Age 30 hr + 50 min at

various stages of division. In certain spores, particularly in certain cultures, there appears to be a reformation of compound nuclei (figure 11, *b* and *c*). This is probably induced by some growth-hindering influence (Knaysi, 1955).

DISCUSSION

We have not made a special study of the mechanism of the staining method used in this work. It appears, however, that it is based on the ability of the methylene blue solution of pH 3.1 to 3.2 to decolorize certain structures already stained with thionin. In the compound nucleus, where the nucleoplasm contains an apparently high concentration of free ribonucleic acid, neither the nucleoplasm nor the chromatin are decolorized. On the other hand, the chemical changes which accompany the formation of the forespore raise the isoelectric point of the nucleoplasm to a point where it is decolorized by the methylene blue solution, and only the chromatin remains stained. The loss of metachromasy indicates either disappearance of the free ribonucleic acid or its combination with a new substance the formation of which was demonstrated previously by the author (Knaysi, 1952). The behavior of the spore upon germination (Knaysi, 1945; Knaysi and Baker, 1947) shows that the ribonucleic acid had not disappeared. The level of the isoelectric point indicates that the newly formed substance is probably a protein. A recent paper by Foster and Perry (1954) demonstrated amino acid synthesis in sporulating cultures.

The conclusion reached, on the basis of the present work, regarding the distribution of chromatin in the resting spore is in general harmony with the observations of Robinow (1953*b*) on spore sections.

Formation of the endospore. The author has studied spore formation on three different occasions. The first investigation (Knaysi, 1946) was restricted to observations of the process in the living state with the ordinary light microscope. The second one (Knaysi and Baker, 1947) was carried out with the electron microscope on sporangia developed in a nitrogen-free medium. It was concluded from this investigation that the endospore is formed as follows: "Two bodies, or groups of bodies, move to a definite distance from each other; an area, slightly denser than the cytoplasm of the mother cell, appears to grow from each body, or group of bodies, toward the other; the forespore results from the merging of the two areas. We never recognized a forespore containing only one body . . . ". The conclusion from the third investigation (Knaysi, 1952), however, was that only one nucleus is responsible for the formation of the endospore although it was recognized that this nucleus sometimes divides in the forespore stage, resulting in more than one nucleus in the spore. The present work shows that the difference between the two conclusions is only apparent. Since the spore is formed by a compound nucleus, the spore primordium of the early bacteriologists, Knaysi and Baker must have observed in their figures 9, 13, and 20, for instance, the formation of that compound nucleus from primary nuclei, whereas in the third investigation observation began after the formation of the compound nucleus, mostly at the time this nucleus underwent the chemical changes described above. The forespores observed by Knaysi and Baker in their figures 16 to 19 as well as those observed by Knaysi (1952) have a structure closely resembling the forespore structure observed in the present investigation.

Chiefly on the basis of present observations, but also taking into consideration the author's

30 C. Fixed with ethanol. Mounted in acid thionin, then in methylene blue solution of pH 3.1 to 3.2. Note numerous forespores. At *a* to *d* the forespores appear to contain each two parallel, or nearly parallel, primary nuclei.

Figure 5. (54 D-10). Microculture and treatment similar to those of figure 4. Age 24 hr. Spore suspension used to inoculate was made in 0.1 per cent, tryptone solution that had been heated in flowing steam for one hour, then cooled before suspension was made. Forespore *a* contains two unequal primary nuclei. Left-hand side of lower nucleus is about twice as large as the right-hand one, indicating that segregation has not yet taken place in this bead or chromosome. At *b*, the forespore appears to contain 6 beads or chromosomes (3 pairs).

Scale of magnification is the same for all 5 figures.

The numbers and letter in parentheses after the figure number identify the negative in the author's library.

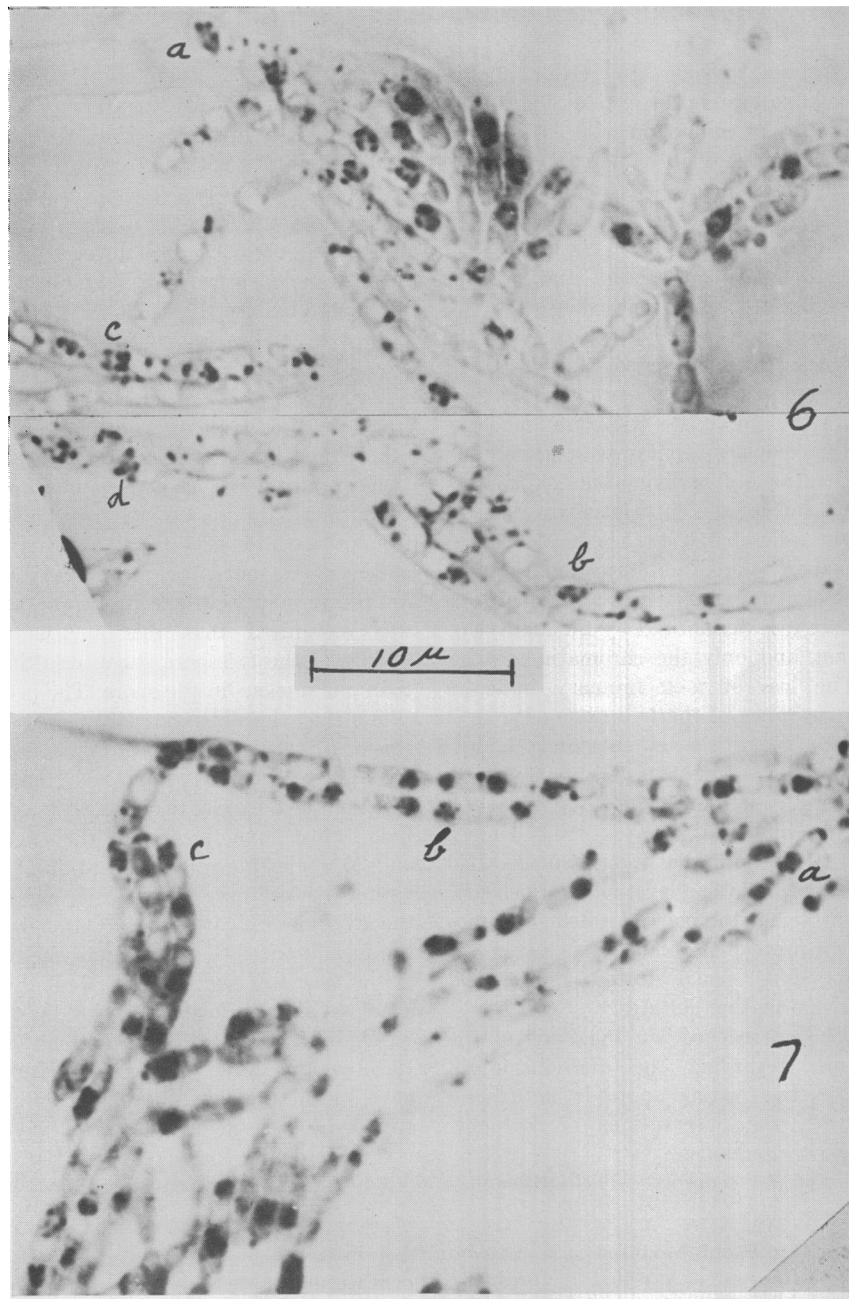

Figure 6. (54 D-4). Same microculture and treatment as in figure 5; a different field showing two parallel, primary nuclei in each of forespores *a* to *c*. At *c*, both primary nuclei consist of one simple and one double bead or chromosomes which appear to have been separated by division. The double bead or chromosome of the upper nucleus is undergoing division (*cf* Knaysi, 1952). At *d*, the pair of primary nuclei form the figure of a cross; here also we note one double and one simple bead in each nucleus.

Figure 7. (54 G-37). Microculture and treatment similar to those of figure 4. Age 16 hr. This figure shows the transition from compound nuclei to forespores. The free ribonucleic acid of the nucleoplasm has not completely disappeared. At *a* and *b*, the embryonic forespores show each a pair of crossed, primary nuclei; at *c*, there are three primary nuclei, two of which are crossed.

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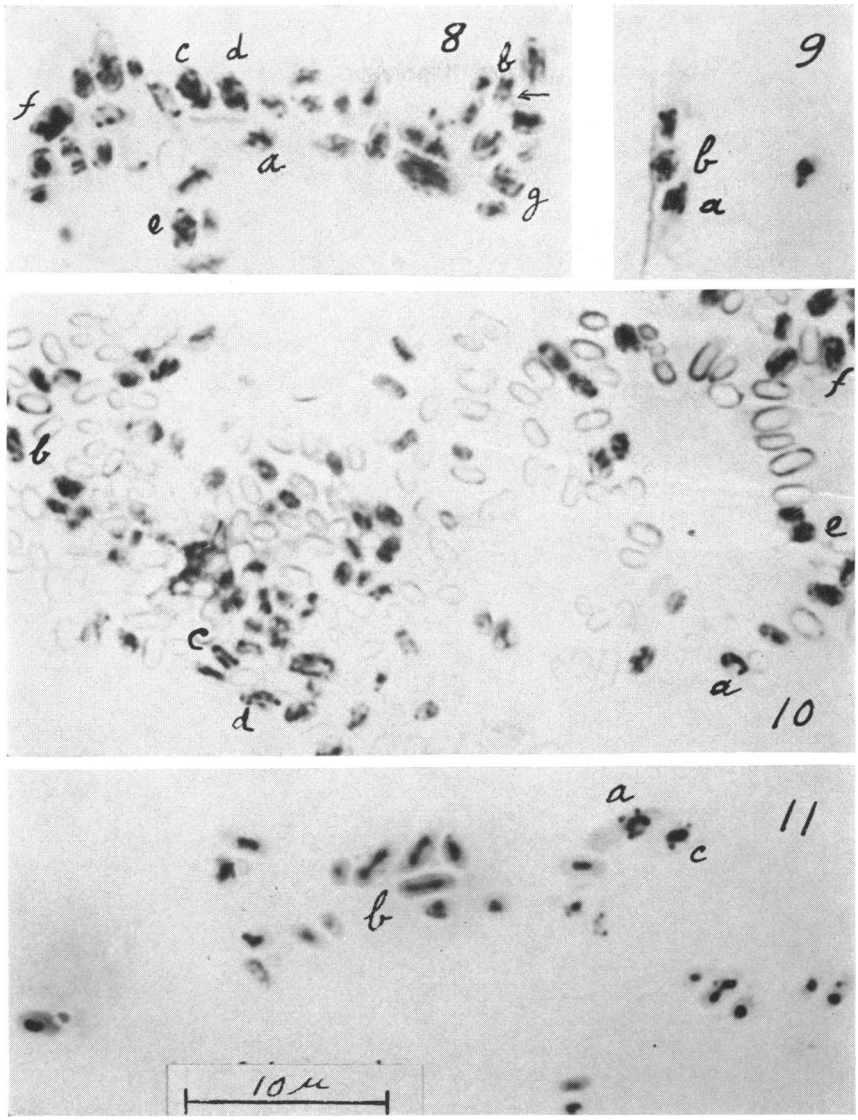

Figures 8, 9, and 11. (54 H-32, 33, and 29). Different fields of the same microculture. Microculture on collodion film supported by MITG/2 agar. Age 2 hr at 30 C. The preparation was first stained with acid thionin, then rinsed, excess free water absorbed, and mounted in methylene blue of pH 3.1 to 3.2. In figure 8, *a* and *b*, the nuclear picture is identical with that observed in the forespores; *c* and *e* show some growth of the primary nuclei; the latter appear homogeneous, indicating increase, but not yet segregation of the chromatin into beads or chromosomes. In *d* the 3 primary nuclei of the spore have divided almost simultaneously, and the daughter nuclei have separated into two groups, one at each pole of the germinating spore. The appearance resembles the stage of late anaphase. *f* represents a less clear picture of an earlier stage. In the lower nucleus at *g* the chromatin has been segregated into two terminal beads, and the nucleus is ready to divide. Figure 9 represents vegetative cells in which the nuclei continue to appear in groups, often resembling certain mitotic figures. *a* and *b* resemble early telophase or late prophase. Figure 11 represents a field in which most of the germinating spores, as at *b*, contained what appear to be compound nuclei; we do not know whether or not these nuclei are genuine or artificially formed by the running together of primary ones. Germ cell *a* shows a group of 3 primary nuclei. At *c*, one sees what appears to be a compound nucleus above a primary nucleus. The terminal beads of the latter resemble centrioles.

Figure 10. (54 I-30). Microculture and treatment as in figure 4. Age 7 hr. At *b*, *e*, and *f*, the germinating spores contain a pair of parallel, primary nuclei each. At *c*, also possibly at *a*, there is one complex primary nucleus in which the chromatin is only partly segregated. At *d*, there are 3 primary nuclei, two of which contain 3 chromatin beads each.

Figures 8 to 11 have the same scale of magnification.

The numbers and letter in parentheses after the figure number identify the negative in the author's library.

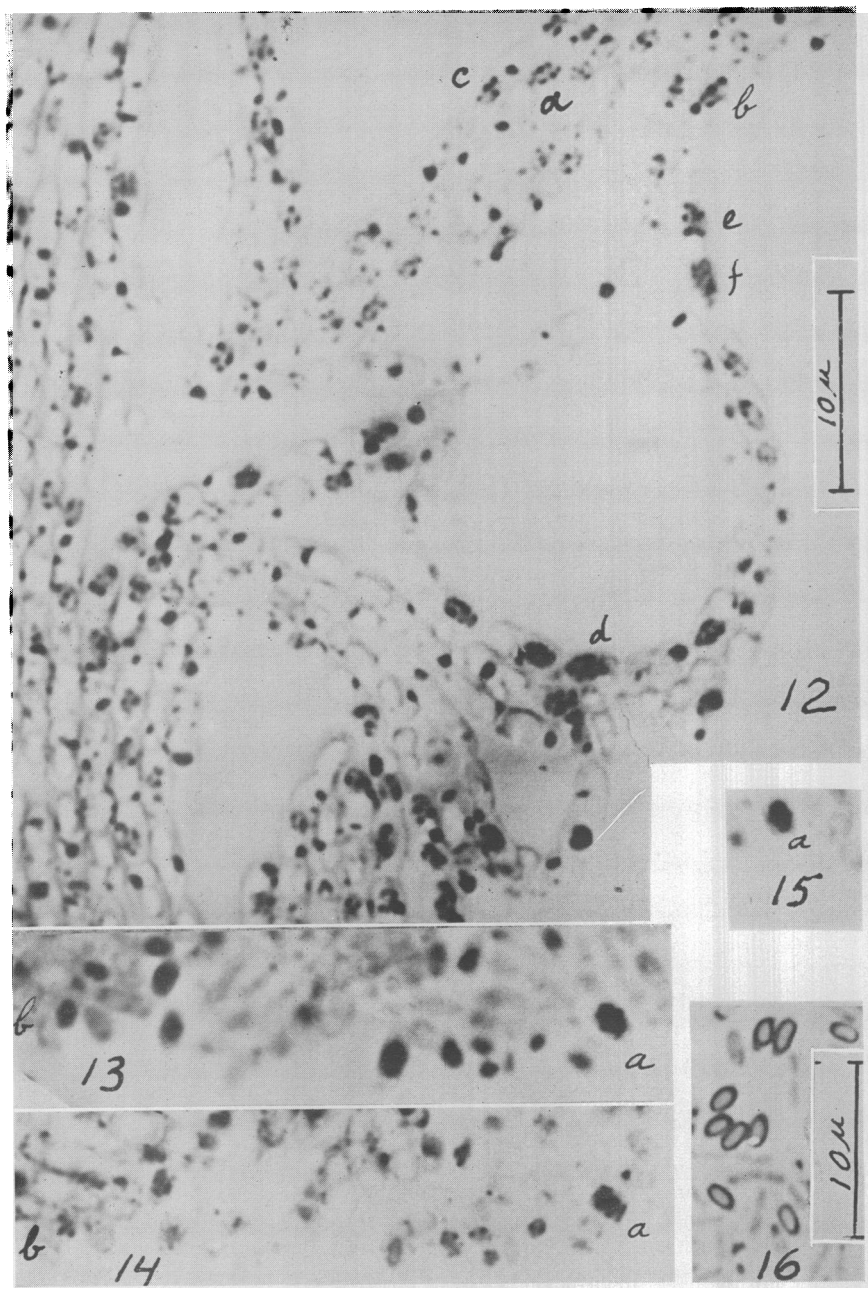

Figure 12. (54 E-21). Same microculture and treatment as figure 4. A different field. Forespore *a* contains either 2 or 3 primary nuclei, one of which has 3 beads or chromosomes. *b* contains two primary nuclei, one of which has 2 and the other 3 beads. *c* probably contains two primary nuclei of 2 beads each. *d*, *e*, and *f* contain more than two nuclei each. *d* probably contains 5, *e*, 3, and *f*, 4 primary nuclei.

Figure 13. (54 G-35). Microculture and treatment similar to those of figure 1. Age 40 hr. Forespores *a* and *b* give the impression of having an equatorial ridge.

Figure 14. (54 G-36). Same microculture as in figure 13 remounted in methylene blue of pH 3.1 to 3.2 after removal of thionin. Forespores *a* and *b* are the same as in figure 13. *a* contains 5, homogeneous, primary nuclei; *b* contains 2 primary nuclei each containing beads or chromosomes. These and other

previous investigations referred to above, formation of the endospore in the strain C_3 of *B. cereus* may be described as follows: A compound nucleus, previously formed by the association of two or more primary nuclei, and usually located at one end of the potential sporangium, undergoes certain chemical changes resulting in the loss of metachromasy and a rise in the isoelectric point of its nucleoplasm. The chief change is probably the synthesis of protein which unites with the ribonucleic acid of the nucleoplasm. These chemical changes begin the forespore stage. The change from a compound nucleus to a forespore does not seem to involve any fundamental modification in structure. The primary nuclei continue to occupy a superficial position, mostly lateral. Previous observations (Knaysi, 1952) of the process of sporulation on living cells indicated an intense synthetic activity during the forespore stage, resulting in some increase in the dimensions of the forespore, particularly in its length. Sometimes one or more of the primary nuclei divide (figure 6, *c*). The forespore stage is terminated by the formation of a dense coat in the superficial layer, but within the boundary of the forespore. This results in the presence of two coats; the outer one appears to consist of unchanged forespore material and is stainable with neutral and basic dyes (figures 10 and 16); the inner one is the dense, unstainable one characteristic of the spore. The fact that during the early stages of germination the internal structure of the spore is similar to that of the forespore shows that no change in internal structure takes place after the coat is formed and the forespore changes into a spore.

Biological nature of the endospore. With the exception of Schaudinn (1902, 1903), bacterial cytologists have considered the endospore as containing a haploid nucleus. Allen, Appleby, and Wolf (1939) considered the endospore to be usually haploid. "On the other hand some spores appear to be formed . . . without previous meiosis, and it is thought that in these spores, which would be diploid, the meiosis which was repressed at spore formation occurs either when the liberated spores are sown on a fresh medium, or when they are left for some time in an old medium". The present work shows that the forespore is formed by a compound nucleus of variable complexity, formed mostly by the association of two, sometimes three or four, rarely six primary nuclei (figures 12, *d*; 14, *a*). We do not believe that there is the usual fusion of the chromatin in the compound nucleus, and we are inclined to believe that the primary nuclei preserve their identity in the compound nucleus. They range themselves end to end at the periphery, and, at best, terminal anastomosis may occasionally take place. We are even inclined to believe that the long, beaded, primary nucleus observed in certain compound nuclei and forespores results from growth and division of the chromosomes without division of the nucleus, rather than from end to end anastomosis. Nor does fusion take place in the spore or during germination. As the spore germinates, each primary nucleus it contains begins to grow and divide, moving toward the center of what was the nucleoplasm of the compound nucleus and has now become the cytoplasm of the germ cell. The progeny of the primary nuclei of the endospore are ultimately distributed in various vegetative cells, independently or associated with other nuclei of their own or different ancestry. Thus ploidy here does not have the meaning as in higher plants and animals. It merely means a temporary state of isolation or association of primary nuclei in a complex nucleus or as a complex primary nucleus.

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forespores in the figure indicate that the effect of having an equatorial ridge is caused by a cross position of at least one of the primary nuclei of the forespore.

Figure 15. (54 G-38). Same microculture as in figures 13 and 14 after treatment with N HCl for 6 min at 60 C and restaining with Giemsa's solution diluted 5 times. Forespore *a* is the same as *a* in figures 13 and 14. Note shrinkage of the forespore caused by the acid treatment. Note again that the internal structure of the forespore is not revealed by this method.

Figure 16. (50 D-16). A gram negative member of *Bacillus*. Microculture on collodion film supported by a medium consisting of 0.01 g of yeast extract powder + 2 g of agar + 100 ml of distilled water. Age 24 hr at 35 C. Stained with Giemsa's solution without previous treatment with acid. Note the deeply stained outer coat.

Figures 12 to 15 have the same scale of magnification.

The numbers and letter in parentheses following the figure number identify the negative in the author's library.

On the basis of the observations discussed above, the endospore is to be considered as a unique spore, certainly not a sexual spore in the usual sense. Consequently, we find no justification for any change in the terminology currently used in relation with the endospore.

The present investigation indicates that the wall of the germ cell is formed during germination.

Until more strains will have been investigated, we prefer to confine our conclusions to strain C_3 of *B. cereus*.

SUMMARY

The formation, germination, and structure of the endospore of strain C_3 of *Bacillus cereus* were investigated by a new technique.

The endospore is formed by a compound nucleus located in a terminal region of the potential sporangium. The change of this compound nucleus into a forespore is accompanied by a loss of the metachromasy and a rise in the isoelectric point of the nucleoplasm. The change from compound nucleus to forespore apparently is not accompanied by structural reorganization. The primary nuclei, most commonly two, continue to occupy peripheral positions. The unstainable coat typical of the endospore is formed within the boundary of the forespore, so that it is usually surrounded by a stainable layer of forespore material which constitutes an outer coat. The internal structure of the endospore is similar to the structure of the forespore. Upon germination the primary nuclei grow and divide and move toward the center of what was the nucleoplasm of the compound nucleus, and which now becomes the cytoplasm of the germ cell.

Careful observation of the formation and germination of the endospore does not reveal any true sexual process but only a temporary association of primary nuclei which regain their independence during germination.

The wall of the germ cell is probably formed during germination.

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